Novel Hepatoselective Insulin Analogues: Studies with Covalently Linked Thyroxyl-insulin Complexes

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To explore the possibility that insulin analogues designed to have restricted access to peripheral tissues may display relative hepatoselectivity *in vivo*, $N^{\alpha B1}$-thyroxyl-insulin (B1-T4-Ins) and $N^{\alpha B1}$-thyroxyl-aminohexanoyl insulin (B1-T4-AHA-Ins) were synthesized. These insulin analogues bind thyroid hormone binding proteins to form high molecular weight complexes. Effects of intravenous infusions of B1-T4-Ins; B1-T4-AHA-Ins; combined thyroxine binding globulin (TBG) and B1-T4-Ins and combined TBG and B1-T4-AHA-Ins were compared with those of insulin infusion in hyperinsulinaemic euglycaemic clamp protocols in anaesthetized beagles ($n = 4$ and $n = 3$ for combined TBG infusions). Glucose turnover rates were measured using D-[3-^{3}H]glucose infusion. With all 5 protocols the rate of glucose disappearance (Rd) was increased and the rate of endogenous glucose production (Ra) decreased from basal level 13.53 ± 0.60 μmol kg^{-1} min^{-1} ($p < 0.05$). Insulin-like activity for Ra and Rd was calculated as the area between the basal values of each variable and the subsequent values plotted graphically against time (AUC). For insulin, B1-T4-Ins, B1-T4-AHA-Ins, combined infusions of TBG + B1-T4-Ins, and TBG + B1-T4-AHA-Ins, respectively, AUC for Rd values were 6.30 ± 0.69, 3.35 ± 0.53, 4.40 ± 0.64, 2.82 ± 0.40 and 3.46 ± 0.95 (mmol kg^{-1}), all analogue infusions being different from insulin ($p < 0.05$). AUC for Rd was further reduced by addition of TBG to B1-T4-AHA-Ins ($p < 0.05$). In contrast the effect of all analogues on AUC for Ra was similar to that of insulin. These observations are compatible with the suggestion that insulin analogues which bind to thyroid hormone binding proteins retain access to hepatic insulin receptors which primarily control Ra. The reduced peripheral insulin-like effect (Rd) could be due to reduced transcapillary access to peripheral insulin receptor sites. © 1998 John Wiley & Sons, Ltd.

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Introduction

In non-diabetic subjects with normal pancreatic insulin secretion, the liver is exposed to approximately 40–60 % higher insulin concentrations than peripheral tissues.[1] In contrast, in Type 1 diabetic subjects who receive insulin subcutaneously this balance is disturbed and hepatic and peripheral exposures to insulin are similar.

A number of studies have suggested that in comparison to insulin (M_r 5807), proinsulin (M_r 9390) has less of an effect on glucose disposal (Rd) than on glucose production (Ra), i.e. a relatively hepatospecific profile of activity.[2–4] Peripheral intravenous infusion studies with covalent insulin dimers (M_r 11604) also suggested a hepatospecific profile of activity relative to insulin.[5] These results suggested to us that the molecular size of the dimers and proinsulin relative to insulin could influence access of insulin analogues to peripheral tissues compared to the liver. We propose that exploitation of the peripheral capillary endothelial barrier can allow design of insulin analogues which have the potential to restore the physiological balance of insulin action even when administered peripherally.

Methods

$N^{\alpha B1}$-thyroxyl-insulin (B1-T4-Ins) and $N^{\alpha B1}$-thyroxyl-aminohexanoyl insulin (B1-T4-AHA-Ins) (M_r ~6500) were designed and synthesized. The aim was the preparation of insulin-like peptides with the capacity to bind both to the insulin receptor and, via the thyroxyl-moiety, to thyroxine hormone binding proteins present in plasma

Abbreviations: Ra rate of appearance of glucose, and used to indicate endogenous glucose production (here assumed to be hepatic glucose production), Rd rate of glucose disappearance or uptake by insulin sensitive tissues

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in high capacity. The possibility that binding to the thyroxine hormone binding proteins might obscure the receptor binding site of the insulin moiety was considered and a spacer arm in the B1-T4-AHA-Ins analogue was included to decrease the probability that insulin action would be impaired by binding.

Thyroxine and the $N^{\alpha A1}$ and $N^{\varepsilon B29}$ amino groups of porcine insulin were acetylated with Msc-N-hydroxy-succinimido ester to yield Msc-thyroxine and $N^{\alpha A1}$-$N^{\varepsilon B29}$-bis-Msc insulin. The active esters were coupled and deprotected with 1.0 mol l^{-1} NaOH to yield $N^{\alpha B1}$-thyroxyl-insulin (B1-T4-Ins).

B1-T4-AHA-Ins was prepared by first protecting the amino group of aminohexanoic acid (AHA) using the *tert*-butyloxycarbonyl (Boc) group, in the presence of dicyclohexoylcarbodiimide (DCC). Hydroxybenzoyltriacyl (HOBt), was coupled with $N^{\alpha A1}$-$N^{\varepsilon B29}$-bis-Msc insulin and the Boc group was then selectively cleaved with triflouroacetic acid. Msc-T4 was coupled to $N^{\alpha A1}$-$N^{\varepsilon B29}$-bis-Msc insulin with Msc-thyroxine $N^{\alpha A1}$, and as above was deprotected with 1.0 mol l^{-1} NaOH. After purification with high pressure liquid chromatography (HPLC) the yields for B1-T4-Ins and B1-T4-AHA-Ins were 75 % and 37 %, respectively.

Experimental Protocol

The differential effect of these analogues on glucose Ra (effect on the liver) and Rd (effect in the periphery) were compared *in vivo* using a hyperinsulinaemic euglycaemic clamp[5–8] combined with D-[3-^{3}H]glucose infusions in dogs. The binding properties of B1-T4-Ins and B1-T4-AHA-Ins, *in vitro* suggested that human thyroxine binding proteins exhibited higher binding capacity than dog serum thyroxine binding proteins. In order to increase the capacity of the binding proteins *in vivo*, human thyroxine binding globulin (TBG) was also included in some of the analogue infusions.

The studies, each with two rates of infusion of either insulin or one of the two thyroxyl-insulin analogues (with or without prior incubation with TBG), were performed in random order on four intact, adult, overnight fasted anaesthetized beagles weighing 11–15 kg. Protocols were approved and licensed by the UK Home Office under the Animals (Scientific Procedures) Act 1986. Each complete study comprised five separate 8.5 h experiments with an interval of at least 4 weeks between each. After premedication with 0.2 ml Acepromazine (2 mg ml^{-1}, C-Vet Ltd, Suffolk, UK) administered subcutaneously, anaesthesia was induced with a rapid intravenous injection (4 mg kg^{-1}) followed by a continuous infusion (0.075–0.2 mg kg^{-1} min^{-1}) of propofol (Diprivan, ICI Pharmaceuticals, Cheshire, UK) into a lateral saphenous vein of a pelvic limb using a Harvard pump (Harvard Instruments, Massachusetts, USA). Body temperature was maintained at 37 °C with a heating pad. Cannulae (Viggo Products, Sweden) were inserted into the external jugular vein for blood sampling, and the cephalic vein of the thoracic limb for infusion of D-[3-^{3}H]glucose, insulin or thyroxyl-insulin analogue, and dextrose.

A priming dose of D-[3-^{3}H]glucose (25 μCi) (purified by HPLC; Radiochemical Centre, Amersham, UK) followed by a continuous infusion (0.25 μCi min^{-1}) was administered with an infusion pump (Palmer, UK) from 0 min to 510 min. After 120 min to allow equilibration of the glucose tracer, 3 baseline samples were taken at 10-min intervals (120–150 min). A continuous, two-step infusion of insulin or one of the two thyroxyl-insulin analogues was started at 150 min. Insulin or the analogue for infusion was diluted in 0.9 % (w/v) sodium chloride containing 2 ml of the dog's own blood. Infusion rates of insulin or the analogues were 3.4 pmol kg^{-1} min^{-1} for 2 h (150–270 min) followed by 17.2 pmol kg^{-1} min^{-1} for a further 2 h period (270–390 min).

The binding properties of the two semi-synthetic analogues, B1-T4-Ins and B1-T4-AHA-Ins, to human and dog plasma proteins were characterized *in vitro* by use of a fast protein liquid chromatography (FPLC) system. The binding studies *in vitro* suggested that human serum binding proteins exhibited higher binding capacity to both thyroxyl-insulin analogues than dog serum binding proteins. In order to increase the capacity of the binding proteins *in vivo*, it was decided to include studies in which the analogues were incubated with human thyroxine binding globulin (TBG) in dog serum prior to infusion into the dogs.

The combined analogue + TBG infusion mixture was prepared 24 h before the study in 50 ml of the dog's own serum. Human TBG (Boehringer Mannheim GmbH, Germany) 3.3 mg (61 nmol) was added, such that the molar ratio of the analogue to TBG was 1:1.45. The hyperinsulinaemic euglycaemic clamp protocol combined with D-[3-^{3}H]glucose infusion was performed as described above. These studies will be referred to as combined B1-T_4-Ins or B1-T_4-Aha-Ins + TBG infusion studies ($n = 3$).

Between 120 and 150 min four blood samples were drawn at 10-min intervals for immediate determination of basal plasma glucose specific activity, plasma glucose and hormone concentration. After the initiation of the insulin or analogue infusion (150 min), blood samples were drawn at 5-min intervals (until 510 min) for serum glucose determination. An infusion of 20 % (w/v) D-glucose in water with D-[3-^{3}H]glucose (2.5 μCi g^{-1}) was adjusted in order to maintain euglycaemia and glucose specific activity at near basal levels.[8] Blood samples were also collected every 30 min and at 10-min intervals from 240–270 min and 360–390 min for determination of hormone and glucose concentration and the plasma glucose specific activity. Insulin or analogue infusion was discontinued at 390 min. Blood sampling was continued every 5 min to 410 min and at 10-min intervals thereafter for the measurement of plasma glucose specific activity and insulin or total analogue concentration. Samples were taken at 150, 270, 390, and 450 min for

FPLC analysis and at 60-min intervals for the measurement of non-esterified fatty acid (NEFA) concentrations. Serum and plasma samples were stored at −20 °C until assayed.

Analytical Procedures

Insulin concentration in serum samples and the infusion mixtures were determined by double antibody radioimmunoassay as previously described.[9] Thyroxyl-insulin analogue concentration in serum samples and in the FPLC fractions were determined by a double antibody immunoassay with some modifications. The measurement of the concentration of thyroxyl-insulin analogues in the samples was performed in the presence of 8-anilino-1-naphthalenesulphonic acid (ANS) and in barbitone buffer (pH 7.4) to ensure the displacement of the bound ligand from the binding proteins in the samples. The RIAs were performed using the appropriate standard and ^{125}I-ligand.

Glucose concentration in the plasma samples was determined by a glucose oxidase method using the Clandon glucose analyser (YSI Instruments, Ohio, USA). NEFA concentration was determined by the extraction method[10] and a fluorescein assay[11] on an Aminco-Bowman Spectrophotofluorometer (American Instruments Co.).

FPLC Separation of the Samples

The B1-T4-Ins and B1-T4-AHA-Ins analogues ($M_r \approx 6000$) bind to thyroxine-binding proteins ($M_r \approx 54\,000$–$66\,000$ Da). On the basis of molecular weight the free and bound forms of these materials were separated by exclusion chromatography or Fast Protein Liquid Chromatography (FPLC) (Pharmacia FPLC system, Pharmacia LKB Ltd, UK) using a Suprose 12 HR 10/30 column equilibrated with 0.1 mol l^{-1} phosphate buffered saline (PBS) buffer, pH 7.2. The column was eluted with 0.5 ml min^{-1} of PBS buffer, with the column pressure at 1.2 MPa and fractions of 0.35 ml collected by a programmable fraction collector (Pharmacia LKB Ltd, UK). The system was standardized using molecular markers.

To determine the status of the thyroxyl-insulin analogues (bound or free) in the serum samples from infusion studies, the samples (150, 270, 390 and 450 min) were subjected to FPLC separation on the day of the study. The sample (200 μl) was filtered through a syringe filter (pore size 0.2 μm) (Gelman, UK) and applied to the FPLC system. The eluate was collected in human serum albumin coated tubes and the concentration of the analogue in the fractions was measured by RIA. Plasma protein bound and free, i.e. bound and unbound components of analogues and Ins were calculated as a percentage of the total immunoreactive insulin or analogue detected in the elution volume from 6 to 20 ml after subtraction of background. The bound and free components were eluted in elution volumes 6–16 ml and 16–20 ml, respectively. Immunoreactive insulin in samples from the studies with insulin infusion were mainly (96 % of total IRI in sample) eluted in elution volume 16–20 ml.

Calculations

Glucose Ra and Rd at all points were calculated using the two compartment model proposed by Mari,[12] which was modified for inclusion of D-[3-^{3}H]glucose in the dextrose infusion.[8] Prior to calculation of glucose turnover plasma glucose concentrations and glucose specific activity time courses were smoothed using Optimal Segments Technique Analysis.[13]

Metabolic clearance rates of the peptides (MCR) were calculated by dividing the infusion rate (pmol kg^{-1} min^{-1}) by peptide concentration (pmol ml^{-1}) during the steady state. Areas between the curve and the extrapolated baseline (AUC) were calculated to quantitate the changes in glucose Ra and Rd in infusion studies.

Statistical Analysis

The statistical significance of changes occurring during, and differences between changes produced by, the insulin and the analogue infusions were determined by two-way analysis of variance (ANOVA). The significance of differences between two means was tested using Fisher's least squares using Number Cruncher Statistical System software (Hintze, Kaysville, Utah). All values are arithmetic mean ± SEM, with p values <0.05 considered statistically significant.

Results

Serum Insulin and Analogue Concentrations

Following infusion of insulin, insulin concentrations were 0.20 ± 0.01 nmol l^{-1} (240–270 min) and 1.21 ± 0.15 nmol l^{-1} (360–390 min), both significantly different from basal (0.07 ± 0.01 nmol l^{-1}) ($p < 0.01$, Figure 1 (a), (b)). At the end of the study, at 510 min, serum insulin concentration (0.08 ± 0.01 nmol l^{-1}) was not different from basal.

Following infusion of B1-T4-Ins and B1-T4-AHA-Ins, total concentrations of B1-T4-Ins were 1.06 ± 0.10 (240–270 min) and 3.45 ± 0.60 nmol l^{-1} (360–390 min) and B1-T4-AHA-Ins were 1.08 ± 0.05 and 6.57 ± 0.60 nmol l^{-1}, respectively (Figure 1 (a)). At the end of the infusion at 390 min total concentration of B1-T4-AHA-Ins was higher than the concentration achieved with B1-T4-Ins infusions ($p < 0.01$).

Following infusion of thyroxyl-insulin analogues combined with TBG and dog serum ($n = 3$), total concentrations of B1-T4-Ins were 0.94 ± 0.16 (240–270 min) and 2.97 ± 0.56 nmol l^{-1} (360–390 min) and B1-T4-AHA-Ins were 1.14 ± 0.09 and 8.76 ± 1.14 nmol l^{-1}, respectively (Figure 1 (b)). At the end of the infusion at 390 min,

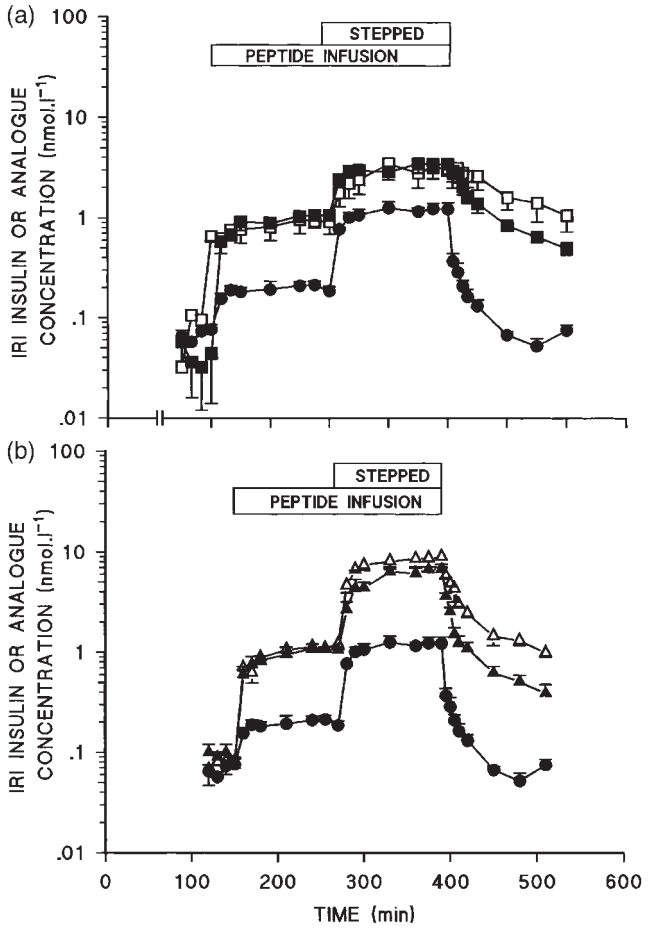

Figure 1. Immunoreactive serum insulin (●) (a) B1-T4-Ins (■) and (b) B1-T4-AHA-Ins (▲) concentrations (nmol l^{-1}) during infusion studies without (closed symbols; $n = 4$) and with TBG (open symbols; $n = 3$). Values are mean ± SEM

total concentration of B1-T4-AHA-Ins was higher than the concentration achieved with B1-T4-Ins infusions ($p < 0.01$).

MCR and $t_{1/2}$ of Insulin, B1-T4-Ins, B1-T4-AHA-Ins and the Combined Infusions

Insulin MCR decreased significantly ($p < 0.05$) with increasing serum insulin concentrations (Table 1). B1-T4-Ins MCR increased significantly ($p < 0.01$) with increasing total serum B1-T4-Ins concentrations (Table 1). Increasing B1-T4-AHA-Ins concentrations had no apparent effect on its MCR.

In combined analogue + TBG infusion studies, B1-T4-Ins MCR increased significantly ($p < 0.01$) with increasing total concentration of B1-T4-Ins. Conversely B1-T4-AHA-Ins MCR decreased significantly ($p < 0.01$) with increasing total serum B1-T4-AHA-Ins concentrations.

Euglycaemic Clamp–Plasma Glucose Concentrations

Plasma glucose concentration was 5.6 ± 0.1 mmol l^{-1} in the basal state, and glycaemia was maintained between 4.5 and 6.2 mmol l^{-1}, in all studies.

Table 1. MCR (ml kg^{-1} min^{-1}) and $t_{1/2}$ (min) calculated from the infusion studies with insulin, B1-T4-Ins and B1-T4-AHA-Ins ($n = 4$) and with combined TBG + B1-T4-Ins and B1-T4-AHA-Ins ($n = 3$)

	MCR (ml kg^{-1} min^{-1})
Insulin	
3.4 pmol kg^{-1} min^{-1}	22.59 ± 0.50
17.2 pmol kg^{-1} min^{-1}	19.47 ± 0.15[a]
B1-T4-Ins	
3.4 pmol kg^{-1} min^{-1}	5.12 ± 0.28
17.2 pmol kg^{-1} min^{-1}	8.40 ± 1.26[a]
B1-T4-AHA-Ins	
3.4 pmol kg^{-1} min^{-1}	7.47 ± 0.90
17.2 pmol kg^{-1} min^{-1}	6.29 ± 1.00
B1-T4-Ins + TBG	
3.4 pmol kg^{-1} min^{-1}	7.05 ± 1.09
17.2 pmol kg^{-1} min^{-1}	10.90 ± 1.37[a]
B1-T4-AHA-Ins + TBG	
3.4 pmol kg^{-1} min^{-1}	8.10 ± 0.69
17.2 pmol kg^{-1} min^{-1}	5.49 ± 0.89[a]

Results are mean ± SEM.
[a]Significantly different from 3.4 pmol kg^{-1} min^{-1} infusion rate, $p < 0.01$.

The area under the curve calculated for exogenous glucose infusion rates (AUC Ginf) (mmol kg^{-1}) to maintain euglycaemia during infusion period (150–390 min) was significantly greater with insulin than with B1-T4-Ins and both combined analogue + TBG infusions ($p < 0.05$), Table 2.

Specific Activity of Plasma Glucose

Specific activity of plasma glucose was maintained at levels similar to basal (98 % ± 3) during the first infusion period (150–270 min) but decreased significantly from the basal (64 % ± 4) with insulin, B1-T4-Ins, B1-T4-AHA-Ins and combined analogue + TBG infusions at 360–390 min ($p < 0.05$).

Glucose Disposal Rates with Insulin and the Analogues

Glucose disposal rates (Rd) significantly increased from basal levels with insulin and the analogues with and without TBG at 240–270 min and 360–390 min ($p < 0.05$; Table 3). At 240–270 min and 360–390 min glucose Rd achieved with B1-T4-Ins, B1-T4-AHA-Ins and both combined analogue + TBG infusions were significantly lower than glucose Rd achieved with insulin ($p < 0.05$). At the end of the study (510 min) glucose Rd achieved with insulin and all the analogue infusions were significantly greater than basal values ($p < 0.05$).

The areas between the curve of glucose Rd and the extrapolated baseline (area under the curve (AUC)

Table 2. Area under the curve (AUC) (mmol kg^{-1}) calculated for (a) glucose infusion rate, (b) glucose Rd and (c) glucose Ra from the infusion studies with insulin, B1-T4-Ins, B1-T4-AHA-Ins and both analogues combined with TBG. The calculated areas were for the area under the infusion period (150–390 min), the period after the cessation of the insulin infusion (390–510 min), and the area under the whole time course of the study (150–510 min); $n = 4$ except for combined studies where $n = 3$

	AUC (mmol kg^{-1}) (150–390 min)	AUC (mmol kg^{-1}) (390–510 min)	AUC (mmol kg^{-1}) (150–510 min)
(a) Glucose infusion rate			
Insulin	8.04 ± 0.95	3.57 ± 0.28	11.60 ± 1.15
B1-T4-Ins	4.89 ± 0.65[a]	4.05 ± 0.47	8.93 ± 1.12
B1-T4-AHA-Ins	6.35 ± 0.52	3.65 ± 0.31	10.00 ± 0.80
B1-T4-Ins + TBG	4.50 ± 0.70[a]	3.73 ± 0.49	8.23 ± 1.76
B1-T4-AHA-Ins + TBG	4.93 ± 1.32[a]	3.18 ± 0.88	8.10 ± 2.19
(b) Glucose Rd			
Insulin	6.30 ± 0.69	2.44 ± 0.26	8.75 ± 0.78
B1-T4-Ins	3.35 ± 0.53[a b]	2.81 ± 0.33	6.16 ± 0.85[a]
B1-T4-AHA-Ins	4.40 ± 0.64[a]	2.45 ± 0.38	6.84 ± 1.00[a]
B1-T4-Ins + TBG	2.82 ± 0.40[a]	2.06 ± 0.30	4.88 ± 0.65[a]
B1-T4-AHA-Ins + TBG	3.46 ± 0.95[a b]	2.05 ± 0.72	5.51 ± 1.67[a]
(c) Glucose Ra			
Insulin	−1.34 ± 0.27	−1.45 ± 0.21	−2.88 ± 0.47
B1-T4-Ins	−1.29 ± 0.38	−1.32 ± 0.28	−2.61 ± 0.64
B1-T4-AHA-Ins	−1.64 ± 0.40	−1.38 ± 0.16	−3.03 ± 0.56
B1-T4-Ins + TBG	−1.64 ± 0.32	−1.83 ± 0.21	−3.47 ± 0.51
B1-T4-AHA-Ins + TBG	−1.04 ± 0.27	−1.17 ± 0.13	−2.21 ± 0.39

Results are mean ± SEM.
[a]Significantly different from AUC calculated from insulin infusions during the same time period, $p < 0.05$.
[b]Significantly different from AUC calculated from B1-T4-AHA-Ins infusions during the same time period, $p < 0.05$.

Table 3. Glucose Ra and Rd (μmol kg^{-1} min^{-1}) achieved with infusions of insulin, B1-T4-Ins, and B1-T4-AHA-Ins, at the end of each infusion period (240–270 min and 360–390 min), without ($n = 4$) and with TBG ($n = 3$)

	Basal	240–270 min	360–390 min
Glucose Rd			
Insulin	14.94 ± 1.11	25.21 ± 1.39[a]	66.1 ± 2.56[a]
B1-T4-Ins	15.06 ± 0.66	18.26 ± 0.84[a b]	51.46 ± 2.62[a b]
B1-T4-AHA-Ins	14.28 ± 1.67	20.31 ± 0.91[a b]	56.24 ± 3.31[a b]
B1-T4-Ins + TBG	16.56 ± 1.11	20.17 ± 1.78[a b]	44.76 ± 2.42[a b]
B1-T4-AHA-Ins + TBG	15.17 ± 1.11	20.47 ± 1.76[a b]	53.10 ± 5.41[a b]
Glucose Ra			
Insulin	14.61 ± 1.05	10.87 ± 0.54[a]	5.79 ± 1.58[a]
B1-T4-Ins	14.83 ± 0.67	8.59 ± 0.49[a]	5.2 ± 0.85[a]
B1-T4-AHA-Ins	13.83 ± 1.67	8.44 ± 0.46[a]	1.31 ± 0.68[a]
B1-T4-Ins + TBG	16.33 ± 1.50	11.08 ± 0.97[a]	2.94 ± 0.93[a]
B1-T4-AHA-Ins + TBG	14.89 ± 1.11	11.87 ± 0.94[a]	7.29 ± 1.23[a]

Results are mean ± SEM.
[a]Significantly different from basal, $p < 0.05$.
[b]Significantly different from glucose Rd achieved with those of insulin infusion at the same period, $p < 0.05$.

for glucose Rd; mmol kg^{-1}) were calculated for each experiment. The mean values and results of statistical analysis are shown in Table 2.

Endogenous Glucose Production Rates with Insulin and the Analogues

Glucose Ra significantly decreased from basal levels with insulin, B1-T4-Ins, B1-T4-AHA-Ins and both combined analogue + TBG infusions, ($p < 0.05$) (Table 3). Glucose Ra achieved with analogues with and without TBG were not significantly different from the glucose Ra achieved with insulin infusion. At the end of the studies (510 min) glucose Ra achieved with all the infusions were significantly different from the basal values ($p < 0.05$).

The areas between the curve of glucose Ra and the extrapolated baseline (AUC), were calculated for each experiment and the mean values are shown in Table 2.

The AUCs calculated for glucose Ra of each analogue infusion at any period were not significantly different from the AUCs calculated for glucose Ra of the insulin infusions.

Hepatic Versus Peripheral Effect

The decrease in glucose Ra (%) versus the increase in glucose Rd (%) (from their own basal levels) achieved with infusions of 3.4 and 17.2 pmol kg^{-1} min^{-1} of insulin, and the analogues without or with TBG are shown in Figure 2 (a) and (b).

The Status of the Thyroxyl-insulin Analogues (Bound or Unbound) in Serum from the Infusion Studies

The IRI in the serum samples from insulin infusion were eluted predominantly between 16 and 20 ml on FPLC (corresponding with the unbound moiety; M_r ~6000 Da). B1-T_4-Ins in the serum samples from infusion studies with B1-T_4-Ins and B1-T_4-Ins + TBG infusions taken at 270 and 390 min eluted mainly between 6 and 16 ml on FPLC (corresponding with bound moiety; M_r ~150 000–12 000 Da). The sample taken 1 h after the end of infusion of B1-T_4-Ins + TBG (450 min) also eluted mainly between 6 and 16 ml (Table 4).

Most of the B1-T_4-AHA-Ins in the samples from infusion studies with B1-T_4-AHA-Ins taken at 270 and 390 min, eluted between 6 and 16 ml (corresponding with the bound moiety; M_r ~150 000–12 000 Da). Chromatography of the samples from the B1-T_4-AHA-Ins + TBG infusion revealed that the presence of human TBG in the infusion had increased the protein bound moiety of the analogue (Table 4) in the serum samples. The sample taken 1 h after the end of infusion (450 min) also eluted mainly between 6 and 16 ml (Table 4).

Non-esterified Fatty Acid Concentrations

There was a significant reduction in non-esterified fatty acid (NEFA) concentration at the end of both infusion periods of insulin (270 and 390 min), but only at the end of the second infusion period (390 min) with B1-T4-Ins, B1-T4-AHA-Ins, and both combined analogue + TBG infusions ($p < 0.05$; Table 5). The NEFA concentration during insulin infusion was significantly different from that achieved with T4-AHA-Ins at 390 min ($p < 0.05$). There were no significant differences between NEFA achieved with insulin and analogue infusions at 270 min. The concentrations of NEFA achieved at 510 min were not different from the concentrations achieved at the end of the infusions (390 min).

Discussion

We have shown that both thyroxine-linked insulin analogues, B1-T4-Ins and B1-T4-AHA-Ins, bind to circulating thyroxine binding proteins in human and dog serum. With concentrations *in vivo* of both analogues and of insulin that suppress glucose Ra equally, there is less stimulation of glucose Rd with the former. The effect is more pronounced when human TBG is added to the infusion mixture.

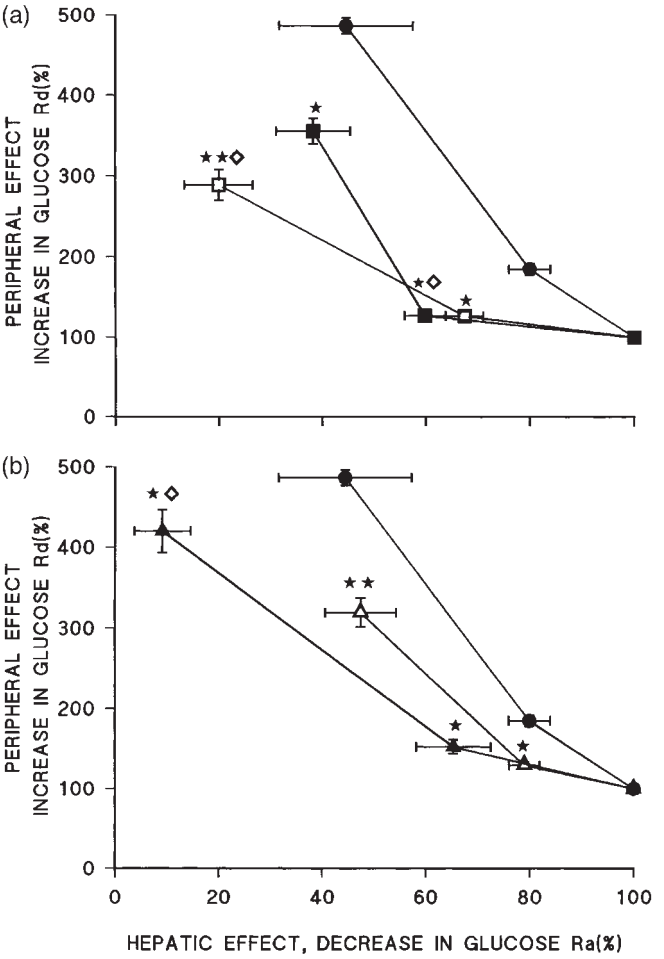

Figure 2. Peripheral effect, increase in glucose Rd (%) versus hepatic effect, decrease in glucose Ra (%) during infusion studies with insulin (●), (a) B1-T4-Ins (■) and (b) B1-T4-AHA-Ins (▲) without (closed symbols; $n = 4$) and with TBG (open symbols; $n = 3$). Results are mean ± SEM. ★ Significantly different from the increase in glucose Rd (%) calculated for insulin infusions during the same time period, $p < 0.05$. ★★ Significantly different from the increase in glucose Rd (%) calculated for the analogue without TBG infusions during the same time period, $p < 0.05$. ◇ Significantly different from the decrease in glucose Ra (%) calculated for insulin infusion during the same time period, ($p < 0.05$)

Despite the fact that it has been demonstrated that some modifications at the B1 position of the insulin molecule do not affect the biological properties of insulin *in vivo* or *in vitro*,[14] addition of the thyroxyl group to the N-terminus of the B-chain reduced the lipogenic activity and receptor binding activity of both analogues compared to insulin *in vitro*.[15] It has been suggested that this could be due to the physical bulk of the thyroxyl group.[15] The spacer arm (amino hexanoyl moiety) of B1-T4-AHA-Ins was introduced to allow access of the insulin

Table 4. Per cent bound to binding proteins and per cent free insulin and analogue immunoreactivity of the samples (150, 270, 390 and 450 min) from infusion studies, after chromatography on FPLC. Total elution volume was 20 ml where the bound and free components were eluted in elution volumes 6–16 ml and 16–20 ml, respectively

	Bound (%)	Free (%)
B1-T4-Ins		
270 min	80.5 ± 2.6	19.7 ± 2.5
390 min	82.4 ± 2.2	17.6 ± 2.2
B1-T4-AHA-Ins		
270 min	60.0 ± 2.4	40.4 ± 1.5
390 min	58.7 ± 5.1	41.3 ± 5.3
B1-T4-Ins + TBG		
270 min	77.5 ± 1.5	22.6 ± 1.9
390 min	89.9 ± 2.5	10.1 ± 2.6
450 min	90.0 ± 4.0	10.4 ± 3.7
B1-T4-AHA-Ins + TBG		
270 min	73.3 ± 1.2	26.0 ± 1.8
390 min	72.9 ± 1.8	27.3 ± 1.7
450 min	85.0 ± 0.4	15.3 ± 0.4

moiety for binding to cell surface receptors and/or to enhance the availability of the thyroxyl moiety for binding to circulating binding proteins. However the data in the present study suggest that either binding to binding proteins did not impair receptor binding or that any impairment so induced was not reduced by the spacer arm.

Metabolic clearance rates (MCR) of chemically modified insulin analogues exhibit saturation kinetics[16–18] and are closely related to their biological responses *in vivo* and their binding properties *in vitro*. The B1-T4-Ins analogue deviated from this rule in that the calculated value of its MCR increased as the analogue concentration was increased during the infusion studies, while the apparent MCR of B1-T_4-AHA-Ins did not fall during the infusion studies. The MCR of total insulin-like growth factor-I (IGF-I) also deviates from this rule[19,20] and has been shown to bind to binding proteins.[21] The increase in MCR with increasing plasma concentration of both thyroxyl-insulin analogues and IGF-I could be explained by the presence of binding proteins which alter the proportion of the peptide which remains unbound. The unbound fraction may be more susceptible to clearance mechanisms than the bound such that, with higher infusion rates and increasing saturation of the binding proteins, the proportion of free peptide (and the MCR) increases. The decrease in the apparent MCR with B1-T_4-AHA-Ins reflects the saturability of the insulin clearance mechanism at increasing serum concentrations and is compatible with its reduced affinity for the binding proteins.

The calculation of the rates of glucose appearance and disposal is heavily dependent on the model used for analysis of the raw data. One-compartment analysis of non-steady state glucose turnover[22] results in a significant underestimation of glucose appearance rates under conditions of high glucose flux in hyperinsulinaemic euglycaemic clamp studies.[23] A two-compartment model[12,24] has been shown to minimize underestimation. In these experiments attempts have been made to clamp the specific activity of plasma glucose to improve precision in the calculation of the rate of glucose appearance.[8,23] The 'spiking' of the exogenous glucose infusion with D-[3-^{3}H]glucose was adjusted for the first infusion rate of each protocol.[8,23] This level of spiking resulted in a reduction of specific activity with the second infusion rate of insulin and the analogues. It has been shown that a fall in specific activity of about 15 % from that in the basal state does not affect the accuracy of measurement of glucose kinetics.[25] The mean glucose Ra calculated from the two pool model at every point during the infusion period was significantly greater than zero. The effect of hyperinsulinaemia on glucose Ra presented here is similar to that reported previously,[8] in which use of exogenous glucose infusion spiked with D-[3-^{3}H]glucose in dogs was associated with incomplete suppression of glucose Ra at similar insulin concentrations.

Insulin and both thyroxyl-insulin analogues exhibited similar effects on glucose Ra (effect on the liver) as calculated from the areas under the curves for glucose Ra during the infusion periods. However, both analogues exhibited a reduced peripheral effect (glucose Rd) in comparison to insulin during both infusion periods. The

Table 5. Non-esterified fatty acid concentration (mmol l^{-1}) during infusion studies with insulin, B1-T4-Ins and B1-T4-AHA-Ins, without ($n = 4$) and with TBG ($n = 3$)

	Basal	270 min	390 min
Insulin	0.65 ± 0.03	0.27 ± 0.03[a]	0.17 ± 0.04[a]
B1-T4-Ins	0.69 ± 0.03	0.45 ± 0.05	0.17 ± 0.02[a]
B1-T4-AHA-Ins	0.76 ± 0.05	0.50 ± 0.11	0.27 ± 0.05[a b]
B1-T4-Ins + TBG	0.82 ± 0.11	0.48 ± 0.12	0.24 ± 0.03[a]
B1-T4-AHA-Ins + TBG	0.81 ± 0.11	0.45 ± 0.08	0.26 ± 0.05[a]

Results are mean ± SEM.
[a]Significantly different from the basal values, $p < 0.05$.
[b]Significantly different from insulin infusions at 390 min, $p < 0.05$.

slow rise in exogenous glucose infusion rate with insulin analogues further suggests that binding to circulating plasma proteins may be successfully limiting access of the analogues to peripheral sites by the endothelial barrier. Data from experiments with proinsulin further support this conclusion.[2,3,26]

Insulin reduces plasma NEFA concentration via its anti-lipolytic action on peripheral fat cells.[27] The effect seen here on NEFA concentrations with insulin infusions demonstrates the sensitivity of the system to insulin and lower infusion rates would be required to demonstrate differential effects at each time point. However our data support the suggestion that addition of a thyroxyl moiety to the B1 group of insulin induces a relative impairment of peripheral but not of hepatic action.

Recently synthetic insulin analogues which rapidly dissociate into monomers have been shown to appear in plasma faster than native insulin from subcutaneous depots. These observations taken together with the data on capillary pore size[28] provide further evidence that the capillary endothelium exhibits a limiting influence on the rate of diffusion of insulin through the interstitial space to the circulation.[29]

It has been suggested that the effects of insulin on glucose production from the liver may be secondary to its peripheral action[25,30] (i.e. an indirect effect). However, glucose output has been shown to be sensitive to minute increases of insulin in the portal vein without and before any effect on glucose Rd.[31,32] Recent studies in conscious dogs[33,34] confirm that both hepatic and peripheral effects of insulin are capable of suppressing glucose Ra. Our data support the conclusion that inhibition of glucose Ra is in part a direct rather than indirect action of insulin.

In conclusion, the observations presented here are compatible with the suggestion that insulin analogues which bind to thyroid hormone binding proteins exhibit easier access to hepatic than to peripheral insulin receptors and that this difference is manifest in their physiological effects on glucose metabolism. Such analogues may offer therapeutic opportunities in the treatment of diabetes mellitus.

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